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Isolation, Identification, and Characterization in Israel of *Brucella melitensis* Biovar 1 Atypical Strains Susceptible to Dyes and Penicillin, Indicating the Evolution of a New Variant

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During characterization by conventional biotyping tests of numerous *Brucella melitensis* isolates obtained in Israel in the last 2 years, we identified several strains of the biovar 1 serotype that are atypically susceptible to dyes and penicillin. Their coisolation from two brothers in a family that rears sheep and from the milk of one of their sheep and the prevalence of such strains in two distinct geographical zones in Israel provide epidemiological support for the notion that a new variant has been identified.

Brucellosis is a zoonotic disease which affects several species of domestic animals commonly reared by humans for the production of milk, meat, and wool. It is transmitted to the human population by direct contact with materials contaminated with the organism or most commonly by the consumption of unpasteurized milk and milk products originating from infected animals. Because of the complications involved in the diagnosis of the disease, including the difficulties in distinguishing between infected and vaccinated animals by conventional serological tests (1, 6), bacteriological isolation and identification of the etiological agent are necessary steps in the design of epidemiological and eradication programs (14).

The current available taxonomy of the genus *Brucella* (2, 5) is only partially adequate for use in the identification and characterization of new isolates. According to the biotyping scheme, the genus is divided into six species, each characterized by distinct host specificity, oxidative metabolism profile, and sensitivity to specific *Brucella* bacteriophages. This scheme, however, does not consider the fact that all the brucellae are closely related by their established high degree of DNA homology (4, 9, 10, 13) and therefore could be regarded as a single species (15, 16). Usually, a consensus upon a classification scheme implicates that the evolutionary development and phylogenetic development of the genus are understood. The inability of the scientific community to define a classification scheme that will best fit the brucellae on one hand and the occasional identification of new, atypical brucella variants that do not fit into the conventional scheme (3, 8, 12) on the other hand may suggest that the genus has not yet reached its final stage of evolution.

Brucella melitensis infection in ovine and caprine herds and in the human population has become a major problem in Israel within the last 2 years. Acting as the national center for the disease, our laboratory is engaged in the isolation and characterization for epidemiological purposes of numerous *Brucella* strains obtained from infected animals and human patients. As *B. abortus* has already been eradicated in our country and swine rearing is not common, *B. melitensis* is the only species prevalent. We report here, for the first time, the isolation, identification, and characterization of *B. melitensis* strains that are serologically designated as biovar 1 and that are atypically susceptible to aniline dyes (basic fuchsin and thionin) and penicillin. Their prevalence in ovine herds and infected human patients possibly indicates the evolution of a new *B. melitensis* variant.

(Part of this research was presented in a poster session at the 8th International Biotechnology Symposium, Paris, France, 17 to 22 July 1988.)

The isolates discussed in this study are described in Table 1. Brucellae from animal sources were isolated in our laboratory by conventional methods (2). Human isolates were received from hospitals nationwide. After strains 86/9413, 87/5361, 87/5968-1393, 87/5968-1394, 87/6012, and 87/10362 were isolated, they were freeze-dried in casein-sucrose-sodium glutamate stabilizer and sent to J. M. Verger and M. Grayon (Station de Pathologie de la Reproduction, Institut National de la Recherche Agronomique, Nouzilly, Monnaie, France) for further characterization. We used conventional biotyping tests to identify and classify the strains (2). The *Brucella* species were determined by the phage typing method with phages Tb and Iz to distinguish between *B. abortus* and *B. melitensis*. Only in the case of the atypical strains were oxidative metabolism tests conducted by J. M. Verger and M. Grayon for characterization. The *Brucella* serotypes were determined with monospecific sera produced in guinea pigs in our laboratory by a standard protocol (2). To distinguish between the *B. melitensis* Rev 1 vaccine strain and field strains, we evaluated their growth in the presence of penicillin, streptomycin, and the dyes basic fuchsin and thionin added at recommended concentrations to tryptic (Difco Laboratories) soy agar. The same tests were also conducted by J. M. Verger and M. Grayon, with the exception of the use of Trypticase (BBL Microbiology Systems) soy agar-serum glucose and Trypticase soy agar supplemented with 0.1% yeast extract as the basal agar media in the dye and antibiotic tests, respectively. In addition, they tested the growth of brucellae in the presence of 100 μg of safranin O per ml. Brucellae were grown in an air atmosphere, unless specified otherwise, by conventional schemes (2).

Only *B. melitensis* isolates were identified in Israel during the last 2 years (Table 2). From these, the isolates allocated to biovar 1 were the most prevalent and those allocated to biovars 3 and 2 were identified less frequently (in that order). In only two incidences had *B. melitensis* infiltrated extensively managed cattle farms. From the biovar 1 isolates, 5 in sheep and 2 in goats were identified as the Rev 1 vaccine

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TABLE 1. *Brucella* strains used in this study

Strain	Description	Reference or source
16M	*B. melitensis* biovar 1 reference strain	2
Rev 1	*B. melitensis* vaccine strain	2
86/9413	Sheep isolate (aborted fetus)	This study
87/5361	Sheep isolate (reproductive organs)	This study
87/5968-1393	Sheep isolate (reproductive organs)	This study
87/5968-1394	Sheep isolate (reproductive organs)	This study
88/3596	Sheep isolate (milk)	This study
89/4429	Sheep isolate (milk)	This study
87/6012	Human isolate	This study
87/10362	Human isolate	This study
88/6717	Human isolate	This study
89/2979	Human isolate	This study

strain and the rest were field strains having either normal (9 in animals and 59 in human patients) or atypical (5 in sheep and 21 in human patients) phenotypes. Chronologically, strain 86/9413 was the first atypical strain identified. More than half a year later we identified strain 87/5361 and, subsequently, strains 87/5968-1393 and 87/5968-1394, which were isolated from three different animals in the same flock. Somewhat later we isolated two additional atypical strains from the milk of infected sheep in two different flocks (Table 1). During this period, we also identified all the atypical isolates obtained from human patients.

The oxidative metabolic tests (Fig. 1) (2) and the phage biotyping tests showed without a doubt that the strains reported here were members of the species *B. melitensis*. Nevertheless, unlike characteristic *B. melitensis* isolates, these strains were atypically susceptible to the standard concentrations of dyes and penicillin used in biotyping tests (Table 3) and to 100 μg of safranin O per ml (J. M. Verger, data not shown), a characteristic which usually distinguishes *B. suis* from the other brucellae. The addition of 10% CO_2 to the growth atmosphere did not change the strain susceptibility. The atypical features of these strains were confirmed as well by J. M. Verger and M. Grayon, except that they could not reproduce our results concerning the susceptibility of the strains to thionin, even when a higher concentration of thionin (40 μg/ml) was used (data not shown). Regarding strains 87/5361 and 87/5968-1393, our data confirmed that they were susceptible to penicillin and dyes, as found for the strains shown in Table 3. However, in J. M. Verger's laboratory, strain 87/5361 was characterized as having a normal phenotype, while strain 87/5968-1393 was found to be only slightly more susceptible to the selective compounds than was a normal type. We attributed the above-mentioned

TABLE 2. *B. melitensis* isolates characterized in Israel from August 1986 through August 1988[a]

Source of brucellae	No. that were biovar:				
	1			2	3
	Normal	Atypical	Rev 1		
Sheep	7	5[b]	5	4	10
Goats	1		2[c]		1
Cows	1				1
Humans	59	21		9	33

[a] Only one isolate per infected flock was included.
[b] Three of the five isolates were from different animals in the same herd.
[c] Isolated in the same herd in two successive years.

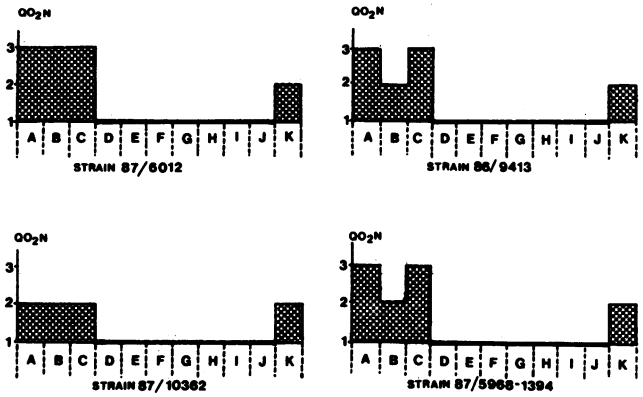

FIG. 1. Oxidative metabolic profiles of the atypical *Brucella* strains. Mean microliters of oxygen taken up per milligram of bacterial nitrogen per hour (QO_2N) level: 1, <100; 2, 100 to 300; 3, >300. Substrates: A, L-glutamic acid; B, L-alanine; C, L-asparagine; D, L-arginine; E, DL-ornithine; F, L-lysine; G, L-arabinose; H, D-galactose; I, D-ribose; J, D-xylose; K, *Meso*-erythritol. The fluctuations observed with substrates A, B, and C have already been observed with *B. melitensis* strains in France (J. M. Verger, personal communication).

differences between the results obtained in the two laboratories to the specific modifications in the composition of the basal agar media used in each location (see above).

Recently, we demonstrated that the atypical strains do cause a zoonotic problem, being transferred from infected sheep to humans. In the last year, two brothers in a family rearing sheep for milk and meat production contracted brucellosis. The *Brucella* isolates obtained from each (88/6717 and 89/2979; Table 1) were characterized in our laboratory as *B. melitensis* biovar 1, having susceptibility features similar to those depicted in Table 3 for the atypical strains. Subsequently, we identified in the herd an infected sheep and isolated brucellae from its milk. This isolate (89/4429; Table 1) also was characterized as *B. melitensis* biovar 1, having atypical features similar to those described above. This result and the fact that a large number of atypical strains (Table 2) have already been identified in

TABLE 3. Biotyping test results for four *Brucella* strains used in this study

Strain[a]	*Brucella* grown in an air atmosphere on[b]:					
	Penicillin G (5 IU)	Streptomycin (2.5 μg/ml)	Basic fuchsin (μg/ml)		Thionin (μg/ml)	
			10[c]	20	10	20
16M	+	−	+	+	+	+
Rev 1	−	+	+	−	+	−
86/9413	−	−	−	−	+	−
87/5968-1394	−	−	−	−	+	−
87/6012	−	−	−	−	+	−
87/10362	−	−	−	−	+	−

[a] All strains were agglutinated in only M antigen-monospecific serum, did not require CO_2 for growth, were lysed by phage Iz at the routine dilution identifying *B. melitensis* but not *B. abortus*, were not lysed by the *B. abortus*-specific phage Tb, produced negligible amounts of H_2S, and had urease activity.
[b] The brucellae were just as susceptible to the antibiotics and dyes when CO_2 was included in the growth atmosphere. +, Normal growth; −, no growth.
[c] Strains 86/9413, 87/5968-1394, and 87/10362 also were susceptible to 5 μg of basic fuchsin per ml (J. M. Verger, personal communication).

Israel, mostly prevailing in only two geographic areas, support the notion that the isolation of the atypical strains was not a mere coincidence but rather indicates that a new variant in the species *B. melitensis* was identified. Since, however, the existence of atypical strains in the species *B. melitensis* has rarely been reported (11, 12), we suggest that they should not form a new taxonomic group. Instead, they should be identified as *B. melitensis* biovar 1 on the basis of their M antigenic serotype and designated atypical with respect to their susceptibility to dyes and penicillin.

It is interesting to note that variant plasticity among *Brucella* species always involves the same very few characteristics of susceptibility of the strains to dyes and penicillin. From the molecular point of view, the existence of these traits in *Brucella* species has recently been shown to correlate with the activity of their outer membrane group 2 proteins as porins (7). It remains, therefore, intriguing to study whether changes in the *Brucella* porins have led to the development of the new variant and what their significance is in the evolution of the genus.

We thank J. M. Verger and M. Grayon for conducting the oxidative metabolic tests and repeating the other biotyping tests required for the identification and characterization of the atypical strains. We also acknowledge their important contribution to the evaluation and discussion of the results. Strain 86/9413 was isolated in our laboratory by M. Bernstein, and the human strains were isolated in Nahariya Hospital and Meir Hospital in Nahariya and Kfar Saba, respectively.

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